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PRINCIPAL INVESTIGATOR: Kevin P. Raisch, Ph.D.

CONTRACTING ORGANIZATION: University of Alabama at Birmingham
Birmingham, Alabama 35294

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6. AUTHOR(S)
Kevin P. Raisch, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Alabama at Birmingham
Birmingham, Alabama 35294

E-Mail:

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We propose to conjugate paclitaxel to a single-chain antibody that specifically binds to the extracellular domain of the EGFR. The scFv-paclitaxel conjugate will be able to localize to the tumor rapidly, penetrate deeper, be eliminated quickly, and induce fewer side effects for the patient, thereby increasing the efficacy of the scFv-paclitaxel conjugate. An additional benefit with the scFv-paclitaxel conjugate is the fact that taxol is a radiosensitizing drug, therefore radiation treatment can be used following the administration of the scFv-paclitaxel conjugate. The EGFR overexpressing breast cancer cell line, MDA-MB-468 will be used in this proposal to show "proof of concept". Cell proliferation assays will be used to examine the cytotoxicity of the scFv-paclitaxel conjugate as compared to paclitaxel, scFv or the combination of the two agents. Cell death will be examined using two different apoptosis assays, annexin V-FITC and TUNEL. Since taxol is a radiosensitizing drug, radiation will be included in the cell proliferation and apoptosis assays. Clonogenic assays will be done to determine the radiosensitizing ability of the scFv-paclitaxel conjugate as compare to each agent alone.

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EGFR-specific single-chain antibody-taxol conjugate for radiosensitizing breast carcinomas

INTRODUCTION

The epidermal growth factor receptor (EGFR) is overexpressed in approximately a third of all breast carcinomas. The overexpression of EGFR has been correlated with a highly malignant phenotype leading to a poorer prognosis for the cancer patients. EGFR-specific mAbs have been used as a carrier for isotopes and toxins to be used for diagnostic and/or therapeutic purposes. We propose to conjugate paclitaxel to a single-chain antibody (scFv) that specifically binds to the extracellular domain of the EGFR. The scFv-paclitaxel conjugate will be able to localize to the tumor rapidly, penetrate deeper, be eliminated quickly, and induce fewer side effects for the patient, thereby increasing the efficacy of the scFv-paclitaxel conjugate. An additional benefit with the scFv-paclitaxel conjugate is the fact that paclitaxel is a radiosensitizing drug, thereby radiation treatment can be used following the administration of the scFv-paclitaxel conjugate. The EGFR overexpressing breast cancer cell line, MDA-MB-468 will be used in this proposal to show "proof of concept". Cell proliferation assays will be used to examine the cytotoxicity of the scFv-paclitaxel conjugate as compared to paclitaxel, scFv or the combination of the two agents. Cell death will be examined using two different apoptosis assays, annexin V-FITC and TUNEL. Since paclitaxel is a radiosensitizing drug, radiation will be included in the cell proliferation and apoptosis assays. Clonogenic assays will be done to determine the radiosensitizing ability of the scFv-paclitaxel conjugate as compare to each agent alone. The successful completion of this project relies on the ability to isolate and purify large enough concentrations of scFvs for the conjugation step with paclitaxel. I have cloned two scFvs which bind to the EGFR. One of the scFvs has been shown to bind to the extracellular domain of EGFR and the truncated EGFRvIII while the second scFv is under investigation. At the end of this granting period (1 year) I have developed the methods necessary to isolate quantities required for the conjugation technique. I will continue this project with the hope of successfully completing this proposal. I will use the preliminary data to expand this grant proposal in which more cell lines will be examined in vitro, the affinity of the scFv will be increased without losing its ability to bind to the EGFR and the scFv-paclitaxel conjugate will be tested in a xenograft mouse model. The scFv-paclitaxel conjugate will be used in combination with radiation, with the hope of lowering the toxicity of taxol without losing any efficacy. The ultimate goal of this project is to translate the results into a clinic trial for breast cancer patients.

BODY

A human IgM scFv phage display library was kindly provided by Dr. Stefan Dübel, University of Heidelberg, Germany. For the library, cDNA was prepared from the peripheral leukocytes of 20 healthy donors as described by Dübel *et al.* (1) using the PCR primer set described by Welschof *et al.* (2). The library, with a calculated complexity of 2 x 10^7 independent clones, was constructed in pSEX81 (Fig. 1) using the *Nco*I and *Not*I restriction enzyme sites (3, 4, 5).

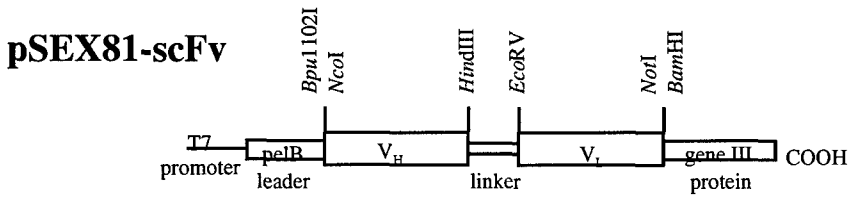

Figure 1. The phagemid, pSEX81, is optimized for surface expression on the M13 bacteriophage. Depicted here is a scFv cloned into the multiple cloning site, in-frame with the pelB leader sequence and gene III M13 protein.

The phage display library was screened as described by Dübel *et al.* (3) using purified EGFR as the antigen. After 3 rounds of phage panning, two individual clones were identified by ELISA (data not shown). The clones, pSEX81-6 and pSEX81-63, have been sequenced and their putative amino acid sequences are shown in Fig. 2. The clones are in the order, variable heavy chain (V_H)-linker-variable light chain (V_L), with both clones containing

a lambda V_L chain (6). When comparing the two clones, there is a 48% amino acid identity in the V_H chain and an 87% amino acid identity in the V_L chain. The hypervariable or complementarity-determining regions (CDRs, as defined by Kabat, 7) are located at the tips of the Fabs in a 3-dimensional structure and have been shown to be primarily involved with antigen binding (6). The CDR1-L region is 100% identical between the two clones, whereas the other CDRs vary from 2 amino acid differences in CDR3-L, CDR2-L and CDR1-H to 10 and 12 amino acid differences in CDR3-H and CDR2-H, respectively. With the high variability between the CDRs of these two clones, I hypothesize that each clone binds to a different antigenic site on the EGFR.

```
     VH→                              [CDR1]              [      CDR2      ]
Clone **** **      ** *   ** *** ******  ******* ***     *    *   **
6    EVQLVESGGGLVQPGGSLRLSCSASGFTFSSYAMHWVRQAPGKGLEYVSAISSNGGSTYYADSVK
63   QVQLVQSGAEVKKPGSSVKVSCKASGGTFSSYAISWVRQAPGQGLEWMGGIIPIFGTANYAQKFQ

                                      [      CDR3      ]              ←VH
     ** **   * *   * *    **** ********  *     **  *     ** *****************
6    GRFTISRDNSKNTLYLQMSSLRAEDTAVYYCVKD..VGGSSWYWADYFDYWGQGTLVTVSSGSASAP
63   GRVTITADESTSTAYMELSSLRSEDTAVYYCARDPDYYGSGSYYPNWFDPWGQGTLVTVSSGSASAP

     [  linker  ] VL→                  [    CDR1   ]                [CDR2  ]
     ************** ******  ***************************** ** **** ***** ** **
6    KLEEGEFSEARVQSVLTQPPSLSVSPGQTASITCSGDKLGDKYASWYQQKPGQSPVLVIYQDRKRPSG
63   KLEEGEFSEARVQSALTQPPSVSVSPGQTASITCSGDKLGDKYASWYQLKPAQSPVWVIYQDTKRSSG

                                  [  CDR3  ]          ←VL
     **** ******** **** ********************  *** *** ********** ***********
6    IPERFSGSNSGNTATLTISGTQAMDEADYYCQAWDSSTPYVFGTGTKVTVLGQPKANPTVTLFPPSSAAA
63   IPERISGSNSGNTSTLTITGTQAMDEADYYCQAWDSSTAVVFGGGTKLTVLGQPKANPSVTLFPPSSAAA
```

Figure 2. Putative amino acid sequence of anti-EGFR scFv clones, pSEX81-6 and pSEX81-63. Clones were sequenced both directions using primers from the pelB leader sequence, the gene III protein and two complementary primers annealing to the alpha tubulin linker sequence. The heavy chain variable region (V_H) and light-chain variable region (V_L) are identified with their respective CDRs (bold) as described by Kabat (7). * identifies identical amino acids.

In order to express, isolate and purify the scFvs, the pSEX81 clones were subcloned into two different vectors. One vector is a bacterial expression vector which will be used to isolate the scFvs which are secreted into the periplasmic space. Both scFv clones were cloned into the bacterial expression vector, pOPE (Optimized for Protein Expression) (Fig. 3). The pOPE-6 and pOPE-63 subclones were shown to express scFvs and the protein was found in the periplasmic space. Studies were done to determine the optimal induction conditions so large quantities of scFv protein could be isolated and purified from the periplasmic space.

The other vector is a eukaryotic secretory vector which will be used to isolate the scFvs which are secreted into the culture medium. To this end, I have subcloned the anti-EGFR scFv clone, pSEX81-6 into the eukaryotic secretory expression vector, pSECTAG/Bpu/neo (Fig. 3). This plasmid was modified by our group in order to accept the scFv clones from the pSEX81 vector using the *Bpu*1102I and *Not*I restriction sites. The scFv was cloned in-frame between the Ig secretory leader sequence on the N-terminal end and two C-terminal tags, myc and $(His)_6$. The anti-EGFR scFv subclone, pSECTAG.6 was stably transfected into the low EGFR expressing human glioma cell line, U87MG (ATCC, Manassas, VA). To isolate stably transfected secreting anti-EGFR scFv subclones, cells were selected for G418 antibiotic resistance and screened the culture medium fr EGFR-positive binding by ELISA. After 3 rounds of subcloning individual cells, scFv-secreting positive sublines were amplified and will be used isolating/purifying scFvs.

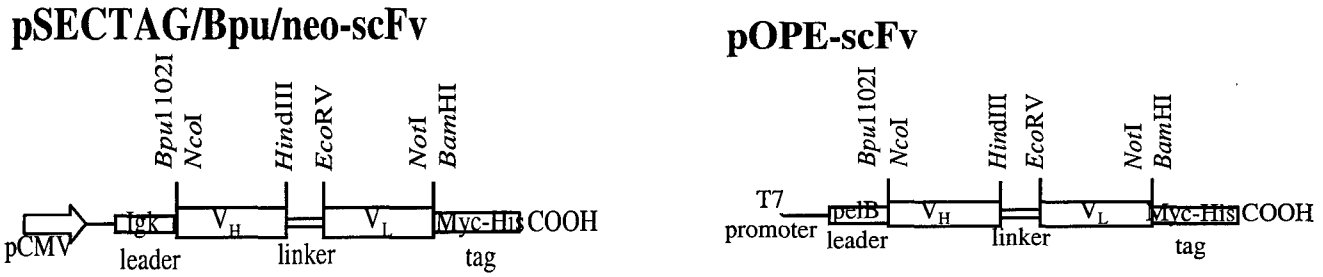

Figure 3. The eukaryotic secreting plasmid, pSECTAG/Bpu/neo was modified from pSECTAG/Friendly (Invitrogen). The neomycin gene replaced the zeomycin gene and a *Bpu*1102I restriction enzyme site was added in-frame with the Ig leader sequence. The bacterial secreting vector, pOPE (Optimized for Protein Expression), uses the pel B leader sequence to target the soluble scFvs to the periplasmic space. Both plasmids contain a myc and $(His)_6$ tag on the COOH end. Each plasmid is shown with a representative scFv cloned into the multiple cloning site.

One of the stably transfected human glioma sublines, U87MG.6.34.A8 (referred to as clone p6.34) was tested for its ability to secrete a functional, anti-EGFR scFv. The scFv was immunoprecipitated from the cell lysate (Fig. 4A) and the culture medium (Fig. 4B) of clone p6.34 but not from the control cell line. The control cells, U87MG.pSECTAG (referred to as clone pSECTAG), are U87MG cells which were stably transfected with the parent vector, pSECTAG/Bpu/neo. We showed that the scFv was translated and processed into the secretory pathway, however the data does not indicate if the clone p6.34 scFv binds to the EGFR antigen. Therefore, an ELISA was performed using the culture medium from the stably transfected cell lines and the same EGFR antigen used for the original scFv isolation procedure. The results shown in Fig. 4C indicate that clone p6.34 secretes scFvs which binds to the EGFR.

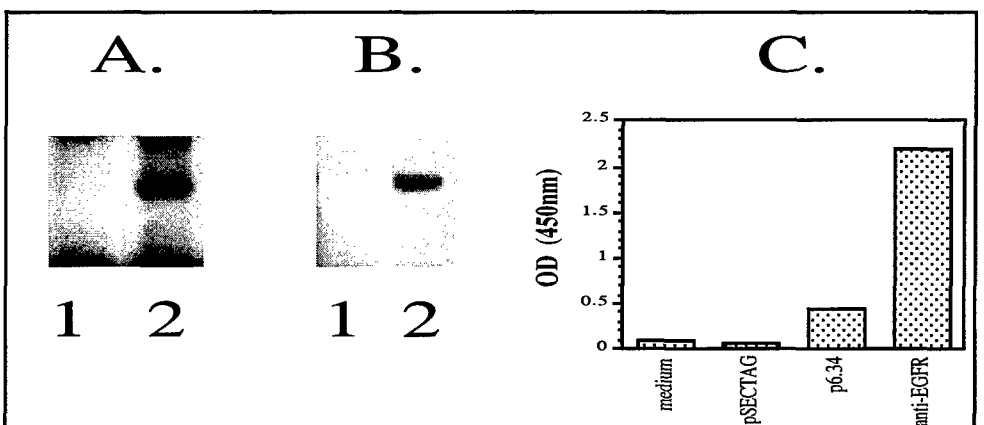

Figure 4. Analysis of secretory anti-EGFR-scFv. A. Immunoprecipitation of scFv from cell lysates, pSECTAG (1) and p6.34 (2) using anti-myc antibody.
B. Immunoprecipitation of secretory scFv from cell culture medium, pSECTAG (1) and p6.34 (2) using anti-alpha tubulin antibody. Anti-myc antibody was used to detect scFvs for both immunoblots, A and B. C. ELISA using cell culture medium, or culture medium from pSECTAG and p6.34, or anti-EGFR (Sigma) to detect scFv binding to the EGFR antigen. ScFvs were detected by anti-myc antibody and developed with OPD.

The ELISA data indicates that clone p6.34 scFv binds to denatured EGFR, however it does provide any information as to which part of the receptor was recognized by the scFv. The EGFR has three major domains, intracellular, transmembrane and extracellular, any of which may serve as the binding site for clone p6.34. To examine whether the scFv binds to the extracellular portion of the receptor, a FACS analysis was used for this determination (Fig. 5). For this assay culture medium collected from 3 different cell lines; U87MG, U87MG.pSECTAG, U87MG.pSECTAG.6.34.A8, was allowed to interact with the cell surface of 3 different human glioma sublines; U87MG, U87MG.wtEGFR and U87MG.ΔEGFR (kindly provided by Dr. H-J. Su Huang, UCSD). The U87MG is the parent cell line into which the scFv clones were stably transfected (this proposal) as well as stably transfected with wild-type EGFR (U87MG.wtEGFR) or the truncated EGFR, EGFRvIII (U87MG.ΔEGFR) (8, 9). An immunoblot indicating the relative densities of EGFR is shown in Fig. 5 (insert) as compared to A431 cells (2 x 10^6 EGFR/cell). The parent cell line, U87MG, has a very low number of

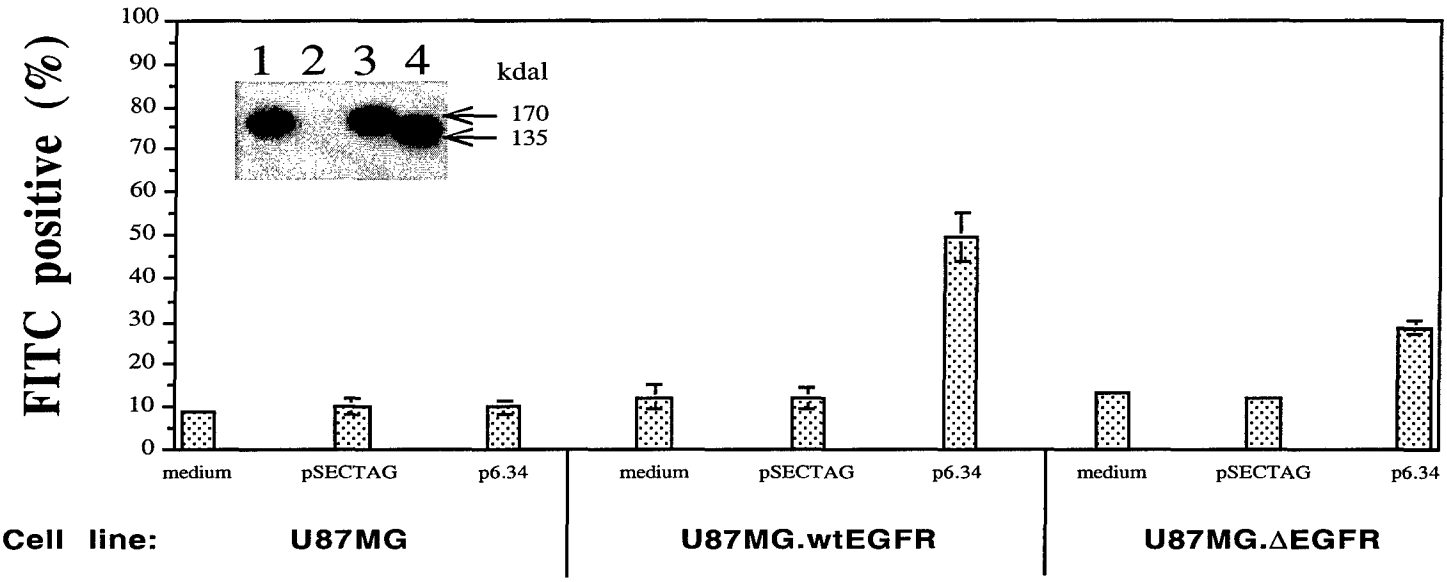

Figure 5. FACS analysis for the detection of anti-EGFR scFvs bound to the extracellular domain of EGFR. The cells, U87MG, U87MG.wtEGFR and U87MG.ΔEGFR were incubated with culture medium, culture medium from pSECTAG or culture medium from p6.34 for 30 min at 4 °C. The cells were washed, then incubated with rat anti-alpha tubulin followed by FITC-labeled anti-rat. Cells were analyzed at the UAB FACS core facility. **Insert.** Detection of EGFR by immunoblot. Cell lysates from 1. A431, 2. U87MG, 3. U87MG.wtEGFR and 4. U87MG.ΔEGFR were separated by SDS-PAGE and transferred to a nylon membrane. EGFR (170 kdal) or truncated EGFR (135 kdal) were detected by anti-EGFR antibody (Sigma) and developed by chemiluminescence.

EGFR, which is one reason why cell proliferation of the stably transfected cell line, clone p6.34, does not appear to be affected by the anti-EGFR scFv (data not shown). The U87MG.wtEGFR subline appears to overexpress a similar number of EGFR/cell as the A431. Of note, the U87MG.ΔEGFR expresses the 135 kdal truncated EGFR which is constitutively phosphorylated (9).

The FACS results shown in Fig. 5 indicates that the secretory scFv p6.34 binds to the extracellular domain of U87MG.wtEGFR and U87MG.ΔEGFR. The parent cell line, U87MG, does not have a significant number of receptors on its cell surface which results in no detectable scFv p6.34 binding. The data indicates that clone p6.34 produced a secretory scFv which bound to the cell surface of cells which overexpress EGFR and truncated EGFR (EGFRvIII). Since clone p6.34 bound to a common antigenic site on both prominent forms of cancer-related EGFRs, this scFv might be able to be used to target paclitaxel molecules to EGFR-overexpressing tumors.

In order to successfully complete this proposal, it is necessary to isolate and purify the scFvs from the U87MG.pSECTAG.6.34.A8 cell culture medium or the periplasmic space of pOPE.clone 6 transformed bacterial cells. I have had much difficulty with this aspect of the project. Initially I used Nickel-columns (purchased from two different companies) to bind the $(His)_6$-tag of scFvs from the culture medium or periplasmic space. The U87MG-clone 6.34.A8 culture medium was used as collected or dialyzed to remove any molecules which might compete with the scFv for the Nickel. Also the proteins from the culture medium were fractionated with ammonium sulfate and the scFv containing fractions (identified by immunoblotting) were collected, dialyzed and used for purification with the Nickel-column. Multiple methods and techniques were attempted but the scFv could not be purified using a Nickel-column. I determined the cause for the inability to purify the scFv was because the scFv would elute off the column with a very low concentration of imidazole (>10 nM) which was included during the column washing steps. Similarly, multiple methods were used to purify the scFvs isolated for the periplasmic space of the pOPE.clone 6 and pOPE.clone 63 transformants.

Recently, I was made aware of a Cobalt-column (Clontech) which binds the $(His)_6$-tag stronger than the Nickel-column. I have been able to elute purified scFvs from the Co-column which are identified by immunoblotting. To-date I have been able to purify scFvs from the cell culture medium and the bacterial periplasmic space.

However, I have not been able to purify the quantity (0.5-1 mg) necessary for Dr. Safavy to conjugate paclitaxel to the scFvs. Currently I am scaling-up the growth of the scFvs and hope to isolate enough protein for the conjugation steps. Then I will be able to proceed with this project to determine if a scFv-targeted taxol molecule will show enhanced breast tumor cell killing as compared to taxol alone.

In order to show proof for this concept, Dr. Safavy and I presented a poster for targeting taxol to EGFR-overexpressing tumor cells by conjugating paclitaxel to the anti-EGFR antibody, IMC-C225 (ImClone Systems, Inc. New York). The following is a brief description of this poster presented at the Radiation Research Meeting (10) and a manuscript is currently in preparation.

Preclinical and clinical studies have shown that the humanized anti-epidermal growth factor receptor (anti-EGFR) antibody, IMC-C225 (ImClone Systems, Inc.), has enhanced antitumor effects of radiation and/or chemotherapy in tumors expressing EGFR. Paclitaxel (PTX) currently shows exciting preclinical efficacy for squamous cell tumors (SCCa). Therefore, we have covalently conjugated PTX to IMC-C225 antibody for targeted drug delivery to EGFR-overexpressing tumor cells. Using MALDI mass spectroscopy the structure and number of PTX molecules per antibody were identified. IMC-C225 was shown to have a mass of 152,387 and the IMC-C225-PTX conjugate was shown to have a mass of 154,189 (Fig. 6). Since paclitaxel has an approximate mass of 900, the conjugate was shown to contain 2 paclitaxel molecules per antibody molecule.

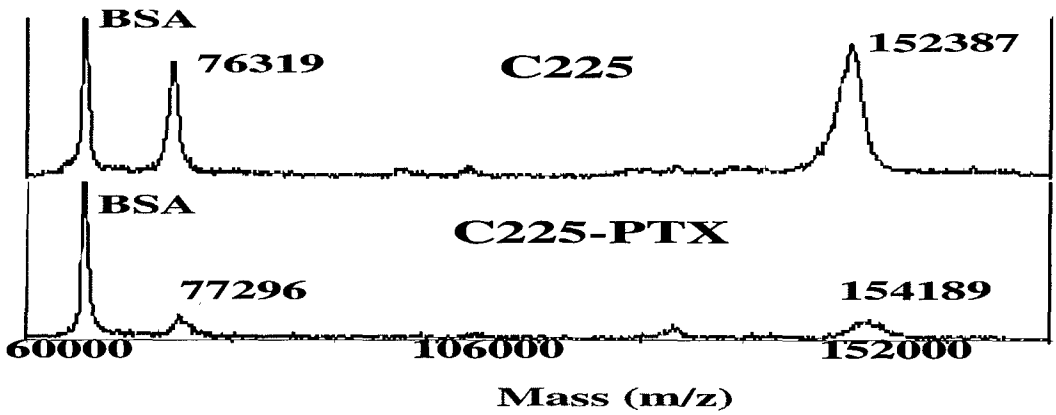

Figure 6. IMC-C225 (C225) was conjugated to paclitaxel (PTX) using a method described previously (Safavy, Raisch et al. 1999). Briefly, the antibody was buffer-exchanged in 50 mM PBS, pH 8.1, and the solution was chilled to 0 °C. Paclitaxel was dissolved in dry DMF under argon and cooled to 0 °C in an ice bath. A solution of EEDQ in DMF was added to the drug solution and the mixture was stirred at 0 °C for 15 min at which time it was added to the IMC-C225 solution. The conjugate, IMC-C225-paclitaxel (C225-PTX), was purified by dialysis against DPBS and the number of conjugated drug molecules was determined by MALDI MS. BSA was used as an internal standard.

A study was undertaken to examine the effects of the IMC-C225-PTX conjugate on the human SCCa cell line, A431. Immunoblot analysis showed that treatment with IMC-C225 or IMC-C225-PTX did not have any affect on the total amount EGF receptor present on the cell (Fig. 7). However both antibodies were shown to block the binding of EGF to the receptor as shown by reducing the EGFR tyrosine kinase activity. The results indicated that the paclitaxel molecules conjugated to IMC-C225 did not impair the ability of the antibody to bind to the EGF receptor.

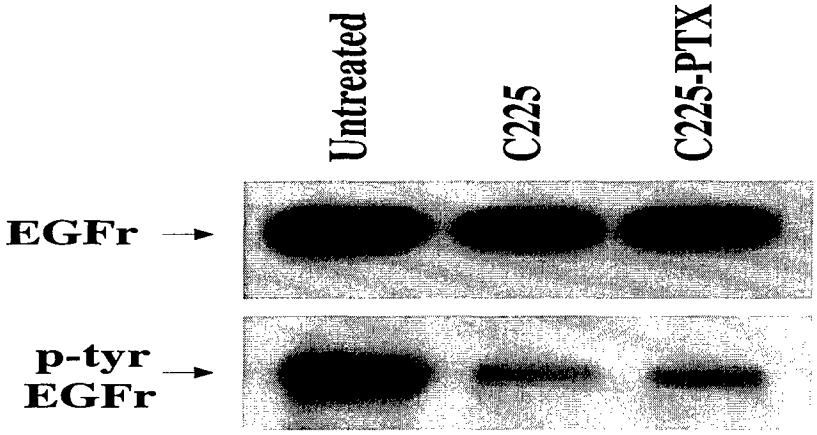

Figure 7. The human SCCa cell line, A431, which overexpress the EGFr (2 x 10^6 receptors per cell), was used in this study. The A431 cells were treated at a concentration of 1 μg/ml with IMC-C225 or IMC-C225-PTX conjugate for 24 h. Then the cells were stimulated with EGF (60 ng/ml) for 5 min. Cell lysates were collected, separated by SDS-PAGE (5 μg/lane) and transferred to a nylon membrane. The immunoblots were probed with anti-EGFr or anti-phospho-tyrosine and developed by chemiluminescence.

Previously, we have shown that IMC-C225 inhibits approximately 50% of cell proliferation at a concentration of 5 μg/ml [33 nM] (11). However using a 24 h exposure to a 10-fold lower concentration of IMC-C225 [3.3 nM] only 8% inhibition of cell proliferation was observed (Fig. 8). PTX alone or IMC-C225 + PTX together showed a 25% and 40% inhibition of cell proliferation, respectively. The IMC-C225-PTX conjugate inhibited 70% of cell proliferation. When the IMC-C225-PTX was combined with ^{60}Co-irradiation there was an 82% inhibition of cell proliferation when compared to the untreated cells.

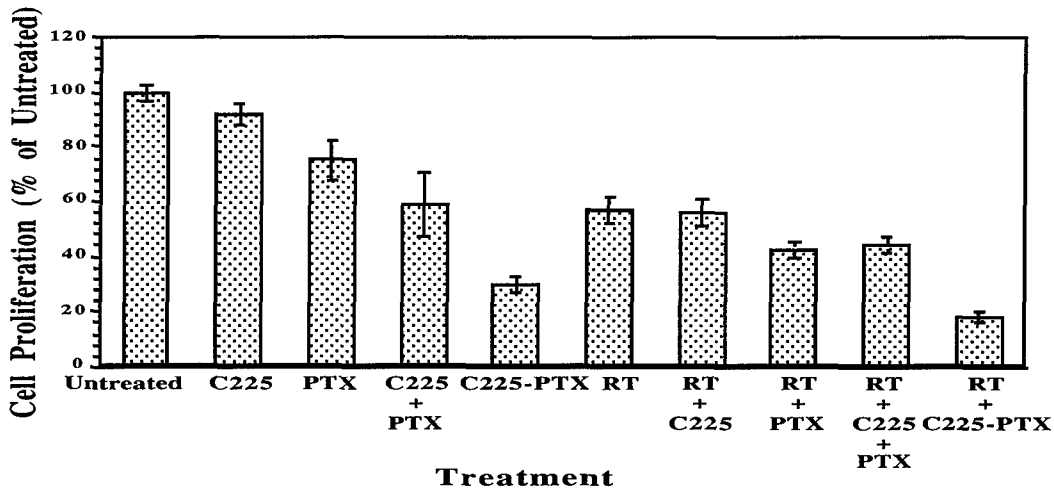

Figure 8. On day 0, A431 cells were treated with IMC-C225 [3.3 nM], paclitaxel (PTX) [6.6 nM], IMC-C225 + PTX, or IMC-C225-PTX conjugate [3.3 nM] for 24 h. At which time (day 1) the cells were exposed to ^{60}Co-irradiation (3 Gy), the treatment was removed and fresh medium was added. On day 4, the cells were counted and normalized to the percent of untreated cells. Each treatment was done in triplicate.

The use of annexin V-FITC and propidium iodide staining to evaluate apoptosis showed no induction of apoptosis by a 3.3 nM concentration of IMC-C225, IMC-C225 + PTX and PTX, whereas the IMC-C225-PTX conjugate induced apoptosis in 28% of the cells and the addition of ^{60}Co-irradiation induced apoptosis in 39% of the cells when compared to the untreated control cells (Fig. 9). Radiation induced apoptosis in 21% of the cells

whereas IMC-C225, PTX or C225 + PTX induced little to no apoptosis either alone or combined with radiation. Comparative analyses revealed that a 5-fold increase in free (unconjugated) PTX concentration was required to induce apoptosis to a similar extent as the IMC-C225-PTX conjugate (data not shown).

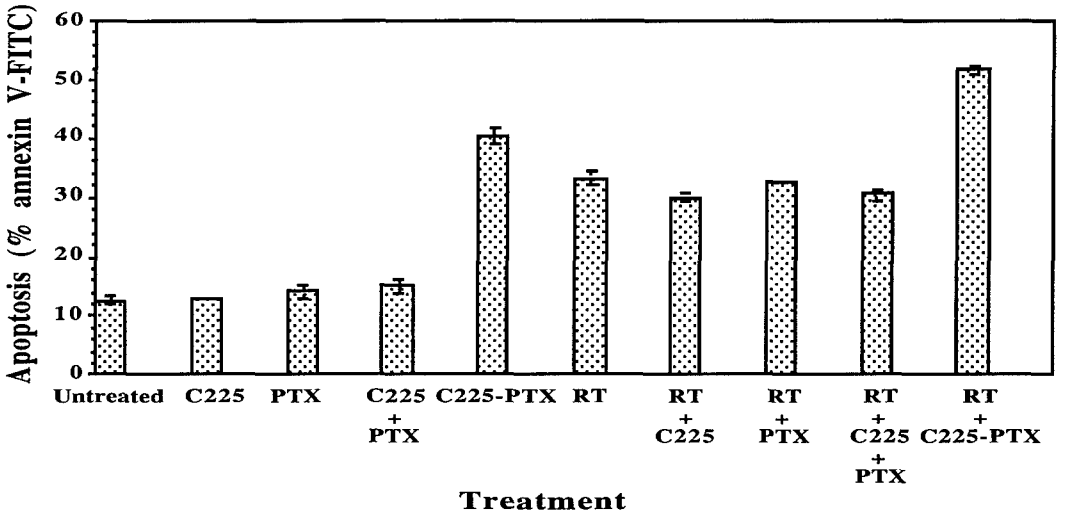

Figure 9. On day 0, A431 cells were treated with IMC-C225 [3.3 nM], paclitaxel (PTX) [6.6 nM], IMC-C225 + PTX, or IMC-C225-PTX conjugate [3.3 nM] for 24 h. At which time (day 1) the cells were exposed to ^{60}Co-irradiation (3 Gy), the treatment was removed and fresh medium was added. On day 4, the cells were analyzed for apoptosis by staining the cells with annexin V-FITC and propidium iodide then sorted by FACS as described previously (11). Each treatment was done in triplicate.

KEY RESEARCH ACCOMPLISHMENTS

-Isolating two human scFvs clones which bind to the EGF receptor
-Showing one scFv binds to the extracellular domain of the EGF receptor and truncated EGFRvIII
-Subcloning scFv clones into eukaryotic secretory expression and bacterial expression vectors
-Perfecting the method for purifying scFv protein using a cobalt column

REPORTABLE OUTCOMES

Received a one year grant from "Friends....you can count on" entitled:
Technetium-99m-labelled human anti-EGFR single-chain antibody for the early detection of breast Cancer (Principal Investigator: Kevin P. Raisch, Ph.D. June 2001-May 2002)

CONCLUSIONS

Approximately 30-35% of breast carcinomas overexpress the EGF receptor, therefore the ability to target taxol molecules to these tumors via a single-chain antibody could show increased survival rates in these patients. I have isolated two scFv clones which bind to the EGFR. One of these clones binds to the extracellular domain of the EGFR and truncated EGFRvIII and could show promise as a targeting agent for taxol molecules. The ability to isolate and purify enough scFv protein for the conjugation process is the major stumbling block for the progression of this project. I have subcloned the scFv clones into two different expression vectors. One vector is used to secrete the scFvs from mammalian cells into the tissue culture medium. The other vector is used to secrete the scFvs into the periplasmic space of bacterial cells. Both expression systems have been shown by

other researchers to be viable sources for functional scFv proteins. After a considerable amount of time spent trying various methods to isolate and purify scFv proteins, I have begun to use a cobalt column which appears to be the most useful method to-date. As the end of this funding period nears, I am isolating larger quantities of scFvs which will be used for paclitaxel-conjugation reactions. Early success of this project will lead to expanding these studies to create an anti-EGFR scFv-paclitaxel conjugate which could be useful in animal models. The affinity of these scFvs are yet to be determined, however site-specific mutations will probably be necessary to make the scFv more useful. Also, the development of diabodies, either with the same anti-EGFR scFv or a second anti-EGFR scFv which also binds to the extracellular domain of the EGFR, would be beneficial in increasing the avidity as well as increasing its overall size for increased serum half-life. We have shown that we can conjugate paclitaxel to full-size antibodies which bind the EGFR and these conjugates do enhance cell killing alone as well as with radiation. Now we must do the same with our scFv proteins.

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APPENDIX

PERSONNEL

Principal Investigator: Kevin P. Raisch, Ph.D.
University of Alabama at Birmingham
Department of Radiation Oncology
1824 6th Ave. South, WTI, room 686
Birmingham, AL 35294
email: raisch@uab.edu
ph. 205-934-0036
fax: 205-975-7060

Co-Investigator: Ahmad Safavy, Ph.D.
Student Assistant: Monika Goyal
Student Assistant: Sakeena Ahmed